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Il Farmaco 56 (2001) 799–802

IL FARMACO

Short Communication

Nitroanilines are the reduction products of benzofuroxan system by oxyhemoglobin (HbO_2^{2+})

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Received 18 April 2001; accepted 23 May 2001

Abstract

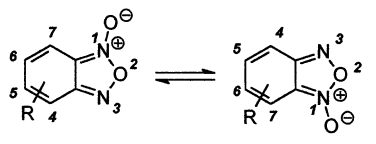
Benzofuroxans are interesting compounds which display several biochemical and pharmacological properties. Recent studies from our laboratory demonstrate that they are reduced by ferrous salts at room temperature and that the principal reaction products are *o*-nitroanilines. This paper shows that simple benzofuroxan derivatives are also able to oxidise HbO_2^{2+} to methemoglobin ($MetHb^{3+}$) (UV detection) and to form *o*-nitroanilines (HPLC detection). From a toxicological point of view this reaction is interesting, since it indicates that the blood is a site for metabolism of these compounds with consequent methemoglobinemia and formation of toxic compounds.

Keywords: Benzofuroxans; *o*-Nitroanilines; Hemoglobin

1. Introduction

Benzofuroxan (benzofurazan oxide) is an interesting ring system whose chemistry has been recently reviewed on various occasions [1–3]. NMR evidence shows that, at room temperature, benzofuroxan derivatives in solution exhibit rapid tautomeric equilibrium **a** ⇆ **b** (Fig. 1).

Thermodynamic equilibrium concentrations are dependent on a number of factors: solvent, temperature, the nature and position of the substituents at the ring. Benzofuroxans display several biochemical and pharmacological properties and a specific review was devoted to this aspect [4]. There are many other possible applications of these compounds [1–3] and worthy of note among them is the use of 'potassium dinitrobenzofuroxan' and of the corresponding barium salt as detonating agents. Recently, we were interested in this heterocycle system as a potential NO donor [5] and for designing new 1,4-dihydropyridines, able either to block or to activate voltage-dependent L-type calcium channels [6,7]. We also studied the reduction of compounds **1**–**3** by ferrous salts at room temperature and we found that the principal reaction products were substituted *o*-nitroanilines [8]. In this paper, we address our study to the interaction of benzofuroxans **1**–**5** with oxyhemoglobin and we show that the reaction is largely comparable with the one with ferrous salts.



a **b**

1 R = H
2 R = 5(6)-CH_3 **3** R = 4(7)-CH_3
4 R = 5(6)-CN **5** R = 4(7)-CN

Fig. 1. Tautomeric equilibrium and studied compounds.

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0014-827X/01/$ - see front matter
PII: S0014-827X(01)01139-9

Table 1
Yields % and reaction times with oxyhemoglobin or $FeSO_4$

Compd.	Yield (with HbO_2^{2+})	Yield of $MetHb^{3+}$	Yield (with Fe^{2+})	Reaction time (with HbO_2^{2+})	Reaction time (with Fe^{2+})
6	89 ± 5	94 ± 3	81 ± 2	60 min	10 min
7a	38 ± 2		60 ± 3	60 min	60 min
7b	54 ± 4	92 ± 3	26 ± 1	60 min	60 min
8b	Quantitative	92 ± 6	70 ± 2	60 min	24 h
9a	35 ± 1		10 ± 1	60 min	60 min
9b	54 ± 2	88 ± 5	67 ± 1	60 min	60 min
10b	65 ± 1		10 ± 2	60 min	24 h
11	15 ± 2	94 ± 5	78 ± 1	60 min	24 h

2. Experimental

2.1. General

Derivatives **1** [9], **2**, **3** [10], **4**, **5** [5], and **12** [11] were synthesised according to literature. Melting points were determined in a Büchi 540 apparatus after introducing the sample into the bath at a temperature 10 °C lower than the melting point and are uncorrected. A heating rate of 1 °C min^{-1} was used. The compounds were routinely checked by infrared spectrophotometry (Shimadzu FT-IR 8101M), ^1H and ^{13}C NMR (Bruker AC200) and mass spectrometry (Finnigan-Mat TSQ-700). HPLC analyses were performed with a diode array UV detector (Shimadzu LC10A). Column chromatography was performed on silica gel (Merck Kieselgel 60, 230–400 mesh ASTM). Thin layer chromatography (TLC) was carried out on plates precoated with Merck silica gel 60 F_{254}, with a layer thickness of 0.25 mm. Petroleum ether 40–60 °C was used. Anhydrous $MgSO_4$ was used as drying agent. Solvent removal was achieved under reduced pressure at room temperature (r.t.). Elemental analyses of the new compounds were performed by REDOX (Cologno M.), and the results are within ± 0.4% of the theoretical values. The figures in brackets are standard errors (± SE).

Caution: Arylamines should be treated as potential carcinogens and handled accordingly with protective gloves in a well-ventilated fume hood.

2.2. Synthesis of derivatives *7–11*

A previously published method [8] was used, partly modified as follows: a solution of the appropriate benzofuroxan (1 mmol) in DMSO was added at r.t. to a stirred solution of $FeSO_4 \times 7H_2O$ (6 mmol) in water. The progress of the reaction was followed by TLC, the reaction mixture was poured into water, extracted with ethyl acetate, and purified by flash chromatography (eluent: petroleum ether–ethyl acetate 7:3). Yields and reaction times are reported in Table 1. Analytical profiles of compounds **7a**, **7b**, **8b** [8], **9a**, **9b** [12] are in agreement with literature data. Compound **11** is identical to the one obtained by the alternative synthesis described below (Scheme 3).

2.3. 3-Amino-2-nitrobenzonitrile *(10b)*

M.p. 198 °C (ethyl acetate–petroleum ether). ^1H NMR (DMSO-d_6): δ 7.20–7.23 (m, 1H), 7.31–7.35 (m, 1H), 7.46–7.54 (m, 1H), 7.79 (br, 2H). ^{13}C NMR (DMSO-d_6): δ 106.55, 117.11 (CN), 124.61, 124.98, 126.47, 134.31, 147.03. EI MS; m/z: M^+ 163 (100). *Anal.* ($C_7H_5N_3O_2$): C, H, N.

2.4. Synthesis of 4-benzofurazanylcarboxylic acid methyl ester *(13)*

A mixture of benzofurazan-4-carboxylic acid (0.6 g, 3.66 mmol), thionyl chloride (5 ml, 69 mmol) and a catalytic amount of DMF was refluxed for 30 min and then evaporated in vacuo. Anhydrous methanol (6 ml) was added and the pure solid separated was collected by filtration and washed with water. Quantitative yield, m.p. 102 °C (ethanol). ^1H NMR (DMSO-d_6): δ 4.30 (s, 3H), 7.76–7.80 (m, 1H), 8.29–8.41 (m, 2H). ^{13}C NMR (DMSO-d_6): δ 163.24 (COO), 149.61, 146.44, 137.16, 132.07, 121.67, 119.10, 52.98 (CH_3). EI MS; m/z: M^+ 178 (45), $[M-31]^+$ 147 (85). *Anal.* ($C_8H_6N_2O_3$): C, H, N.

2.5. Synthesis of 4-benzofurazanylcarboxyamide *(11)*

To a stirred ice-water cooled solution of **13** (0.5 g, 2.3 mmol) in methanol (6 ml), an aqueous solution (32%) of ammonia (9.5 ml) was added. The mixture was kept under stirring at r.t. overnight. The solid obtained was filtered off, washed with water and dried to give the pure title product. Yield 53%, m.p. 184 °C (ethanol). ^1H NMR (DMSO-d_6): δ 7.70–7.77 (m, 1H), 7.91–8.03 (br, 2H, NH_2), 8.07–8.10 (m, 1H), 8.23–8.27 (m, 1H). ^{13}C NMR (DMSO-d_6): δ 163.94 ($CONH_2$), 149.38, 146.74, 133.73, 132.40, 123.32, 119.39. EI MS; m/z: M^+ 163 (100), $[M-16]^+$ 147 (55). *Anal.* ($C_7H_5N_3O_2$): C, H, N.

Table 2
Kinetic data

Compd.	ΔA (min^{-1})
1	0.0920 ± 0.0039
2	0.105 ± 0.011
3	0.0137 ± 0.0014
4	0.0884 ± 0.0053
5	0.0171 ± 0.0029

2.6. Preparation of oxyhemoglobin

Pure oxyhemoglobin was prepared by adding an excess of the reducing agent sodium dithionite ($Na_2S_2O_4$) to a solution of commercially available bovine hemoglobin (H-2500 Sigma Chemical Co.), in 50 mM phosphate buffer (pH 7.4), at 4 °C and protected from light. After 5 min the resulting solution was loaded onto a chromatographic column (Sephadex G-25, Pharmacia, Uppsala, Sweden) and eluted with phosphate buffer. The purity of the oxyhemoglobin solution was determined spectrophotometrically ($\lambda_{max} = 414$ nm; $\varepsilon = 125\,000$).

Analogously pure methemoglobin and carboxyhemoglobin (HbCO) were prepared by treating solutions of commercially available bovine hemoglobin with excess of $K_3Fe(CN)_6$ and CO, and then purified by gel chromatography.

2.7. Study of the reaction with oxyhemoglobin

A solution of the appropriate benzofuroxan in methanol was added to 1 ml of 50 mM phosphate buffer (pH 7.4) containing freshly prepared HbO_2^{2+} and 2-nitro-4-chloroaniline as internal standard (IS). The final concentrations were 0.2 mM for benzofuroxans and IS and 0.4 mM (heme) for HbO_2^{2+}. After 1 h, the mixture was diluted 1:1 with acetonitrile, centrifuged (10 min, 9600*g*) and analysed by HPLC, using a Merck Purospher RP-18 column (250 × 4 mm; 5 μm particles) at 40 °C, eluting (1 ml min^{-1}) with 65% aqueous acetonitrile containing 0.1% trifluoroacetic acid. Yields of the reaction products are reported in Table 1.

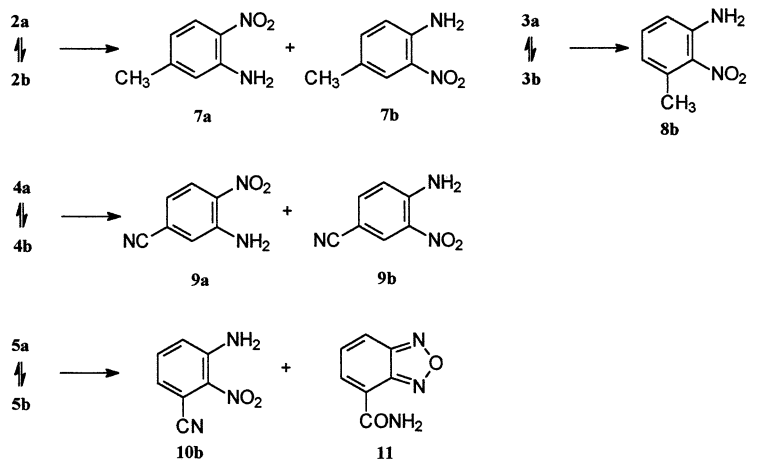
$MetHb^{3+}$ formation in the reaction mixture was determined spectrophotometrically at $\lambda = 630$ nm ($\varepsilon_{MetHb} = 3630$) and 577 nm ($\varepsilon_{HbO2} = 15\,000$) [13].

2.8. Oxyhemoglobin oxidation kinetic study

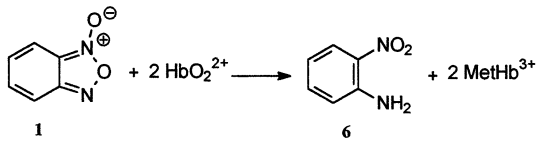
The rate of oxidation was determined using a spectrophotometric technique based on the conversion of HbO_2^{2+} to $MetHb^{3+}$. The formation of $MetHb^{3+}$ was followed by recording the absorbance increase (ΔA at $\lambda = 401$ nm) using a Perkin–Elmer Lambda 5 spectrophotometer and a thermostated (37 °C) glass cuvette. The reaction was started by adding DMSO solutions of the compound (final concentrations 50 μM) to a 4 μM HbO_2^{2+} solution in 50 mM phosphate buffer (pH 7.4). The increase of absorbance was recorded over the first 3 min. The initial oxidation rates expressed as ΔA min^{-1} (Table 2) were calculated from the slope of the straight line portion of each curve. Rate value is the average of at least three determinations.

3. Results and discussion

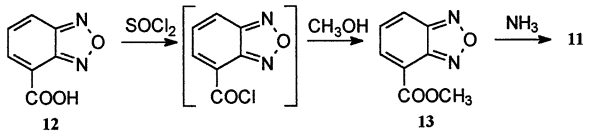
The reaction of benzofuroxans and HbO_2^{2+} was run by treating in pH 7.4 phosphate buffer, at 37 °C, each benzofuroxan derivative with purified oxyhemoglobin in 1:2 molar ratio (benzofuroxan/heme). Analysis of the reaction products showed that HbO_2^{2+} was transformed into $MetHb^{3+}$ and the benzofuroxans into the corresponding *o*-nitroanilines (Scheme 1). $HbO_2^{2+} \rightarrow MetHb^{3+}$ transformation was monitored by UV–Vis spectroscopy, while the formation of nitroanilines was detected by HPLC using the appropriate nitroaniline as standard. After 1 h the reactions were completed by over 90% (Table 1). This means that the reduction of



Scheme 1.



Scheme 2.



Scheme 3.

benzofuroxan system **1** by HbO_2^{2+} involves two electrons (Scheme 2) and parallels the reduction by ferrous salts. The most relevant difference is that the former process is quite faster and the yields are partly different. In some cases with Fe^{2+} ions the reduction was not completed even after 24 h.

Literature data [5] show that both **2** and **3** exist in tautomeric forms (**2a**/**2b** ca. 1:1, **3a**/**3b** ca. 3:1, $CHCl_3$, −40 °C). Reduction by HbO_2^{2+} affords in the case of **2** a mixture of the nitroanilines **7a** (38%) and **7b** (54%). After 24 h the reduction with ferrous salts also gives similar results but the yields in anilines were reversed (**7a**, 60%; **7b**, 26%). Reduction of **3**, both with HbO_2^{2+} and ferrous salts, produces only nitroaniline **8b**. Therefore, in both cases the thermodynamically favoured 4-methyl isomer is selectively less reactive than the 7-methyl one. This is probably due to steric hindrance to electron transfer to N-3 of the benzofuroxan system [8]. Also, compounds **4** and **5** exist in tautomeric forms (**4a**/**4b** ca. 1:2, acetone −20 °C; **5a**/**5b** ca. 5.7:1, acetone, 0 °C) [5]. Reduction of **4** by HbO_2^{2+} parallels the reduction of the corresponding methyl derivative **2** affording the expected mixture of nitroanilines **9a** (35%) and **9b** (54%). Again, different yields are obtained in the reaction with Fe^{2+}. The reduction of **5** gives only partly similar results to those obtained in the reduction of the methyl analogue **3**, since besides the sole nitroaniline **10b** (65%) we isolated in low yield (15%) the benzofurazan derivative **11**, as a result of the combined CN hydrolysis and N→O deoxygenation processes. The structure of **11** was confirmed by direct synthesis (Scheme 3). This same derivative is formed as the principal product in the reaction by Fe^{2+} salts. Reduction of benzofuroxans by oxyhemoglobin does not seem to involve auto-oxidation of the heme group with production of intermediate O_2^-, since the same results were obtained using $HbCO^{2+}$. Initial rates of these reductions, using an excess of benzofuroxan derivatives with respect to HbO_2^{2+}, were measured following the formation of $MetHb^{3+}$ by UV spectroscopy. 5(6)-Substituted benzofuroxans display initial rates similar to that of **1**, while 4(7)-tautomers were significantly slower. This technique is frequently used to detect nitric oxide (NO) under aerobic conditions and to measure initial rates of NO release by NO donors (oxyhemoglobin assay) [14,15]. In fact NO is able to transform HbO_2^{2+} into $MetHb^{3+}$ and nitrate according to equation

$$HbO_2^{2+} + NO \rightarrow MetHb^{3+} + NO_3^-$$

Since benzofuroxans, in these conditions, are unable to release NO [5], this is a good example as the above reaction should be used carefully because potential NO donors can directly promote $HbO_2^{2+} \rightarrow MetHb^{3+}$ transformation.

In conclusion, this paper suggests that blood is a possible site for metabolism of benzofuroxan derivatives with the formation of methemoglobin and toxic *o*-nitroanilines.

Acknowledgements

The financial support from MURST, Rome, is gratefully acknowledged.

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